High prevalence of encoding RhoA-targeting toxin in clinical isolates of Staphylococcus aureus

Patrick Munro, René Clément, Jean-Philippe Lavigne, Céline Pulcini, Emmanuel Lemichez, Luce Landraud

High prevalence of *edin-C* encoding RhoA-targeting toxin in clinical isolates of *Staphylococcus aureus*

Patrick Munro[1], René Clément[1], Jean-Philippe Lavigne[4,5], Céline Pulcini[2,3], Emmanuel Lemichez[1*] and Luce Landraud[1,6*]

Running title: EDIN exotoxins in *S. aureus* infections

1 INSERM, U895, C3M, toxines microbiennes dans la relation hôte pathogènes, Université de Nice-Sophia-Antipolis, UFR Médecine, IFR50, Nice, F-06204, France

2 Université de Nice-Sophia-Antipolis, UFR Médecine, IFR50, Nice, F-06204, France

3 Service d'Infectiologie, Hôpital l'Archet 1, Route Saint Antoine de Ginestière, BP 3079, 06202 Nice Cedex 3, France

4 INSERM, Espri 26, Université Montpellier 1, UFR de Médecine, Nîmes, France

5 Laboratoire de Bactériologie, CHU Caremeau, Nîmes, France

6 Laboratoire de Bactériologie, CHU de Nice, Hôpital l'Archet, Nice, France.

*** Corresponding authors:**

Luce Landraud, INSERM, U895, C3M, toxines microbiennes dans la relation hôte pathogènes, Université de Nice-Sophia-Antipolis, UFR Médecine, IFR50, Nice, F-06204, France, and Laboratoire de Bactériologie, CHU de Nice, Hôpital l'Archet, Nice, France.

Telephone: 00 33 4 89 06 42 61

Fax: 00 33 4 89 06 42 60

Mail: landraud.l@chu-nice.fr

Emmanuel Lemichez, INSERM, U895, C3M, toxines microbiennes dans la relation hôte pathogènes, Université de Nice-Sophia-Antipolis, UFR Médecine, IFR50, Nice, F-06204, France, and Laboratoire de Bactériologie, CHU de Nice, Hôpital l'Archet, Nice, France.

Telephone: 00 33 4 89 06 42 61

Fax: 00 33 4 89 06 42 60

Mail: lemichez@unice.fr

Abstract word count: 173

Abstract *Staphylococcus aureus*, a major causative agent of human infection produces a large array of virulence factor including various toxins. Among them, the host RhoA GTPase ADP-ribosylating EDIN toxins are considered as potential virulence factors. Using the polymerase chain reaction, we analyzed the virulence profile of 256 *S. aureus* isolates from various clinical sites of infections. We developed specific primers to detect the three isoforms of *edin* encoding genes. We found a prevalence of 14% (36 bacteria) of *edin* encoding genes among these clinical isolates. Strikingly, we found that 90% of all *edin*-bearing *S. aureus* isolates carried the type-C allele. Both the *spa* types and the profile of virulence factors of these *edin*-positive isolates are highly variable. Notably, we show for the first time that *edin*-C positive isolates were more frequently recovered from deep-seated infections than other types of infections. Our present work thus strongly suggests that presence of *edin-C* is a risk factor of *S. aureus* dissemination in tissues and thus represents a predictive marker for a pejorative evolution of staphylococcal infections.

Keywords

Staphylococcus aureus, EDIN, toxin, ADP-ribosyltransferase, virulence factors, Rho GTPases.

Introduction

Staphylococcus aureus is a common bacterium, which is responsible for a unique variety of infections [1]. Development of pejorative forms of staphylococcal infections involves the combined action of numerous bacterial virulence factors, which corrupt host responses [2]. Bacterial virulence factors include specific adhesins, collectively referred as Microbial Surface Components Recognizing endothelial cell Adhesive Matrix Molecules (MSCRAMMs) and a large variety of toxins, such as the exfoliative toxins (ETs), hemolysin, leukocidin, enterotoxins and EDINs (epidermal cell differentiation inhibitors) [3-6].

EDINs belong to the family of *Clostridium botulinum* C3 exoenzyme [6, 7]. They are members of a group of major bacterial virulence factors targeting host Rho GTPases [4, 6-9]. Rho proteins control essential cellular processes such as cell polarity, movement and phagocytosis, as well as cohesion of intercellular junctions [6, 10, 11]. Recent findings suggest that EDINs might favor bacterial dissemination in tissues, by a haematogenous route, through induction of large transcellular tunnels in endothelial cells named macroapertures [12-14]. Indeed, recent data show that *S. aureus* EDIN toxin promotes formation of infection foci in a mouse model of bacteremia [15]. To date, three isoforms of EDIN have been characterized. These comprise the first discovered EDIN isoform (EDIN-A), isolated from the E-1 strain of *S. aureus* [16], as well as EDIN-B [6, 17] and EDIN-C [18]. The chromosomal gene encoding *edin-B* is located within a pathogenicity island frequently associated with the *etD* gene encoding the exfoliative toxin ET-D [17]. EDIN-C is encoded by the pETB plasmid, which also carries genes encoding ET-B and conferring cadmium resistance [18].

A first epidemiological survey, involving staphylococcal strains isolated from patients hospitalized for various infectious diseases demonstrated a higher prevalence of *edin*-encoding genes in this group compared to nasal strains isolated from healthy students [19]. Another study shows that *edin-B* is present in 7% of bacteriemic *S. aureus* strains [17]. However, most other epidemiological data on *edin* are based on surveys focused on exfoliative toxins or PVL rather than EDIN toxin itself. For example, a genetic association between *etD* and *edin-B* was detected in the emerging ST80 clone Panton-Valentine Leukocidin (PVL)-positive and community-acquired (CA) methicillin-resistant *S. aureus* (MRSA) [20]. This clone is spreading throughout France and Tunisia and is most frequently associated with infections of the skin and soft tissues. Also, two-thirds of the strains belonging to the emerging ST123 epidemic European fusidic acid-resistant impetigo clone

(EEFIC) were positive for *etB* and sequence analysis of pETB2 (a close homolog of pETB) in one of these strains suggested that it also bears *edin-C* [21].

In this study, we have developed a PCR-based method, to detect EDIN isoforms specifically. We demonstrate that 90% of all *edin*-bearing *S. aureus* isolates carry the type-C allele. We also show that these isolates are more significantly associated with deep-seated soft tissue infections than other types of infections (Fisher's exact test, p=0.03).

Materials and methods

S. aureus isolates

A total of 256 isolates of *S. aureus* belonging to the collection of the Bacteriology department of the Hospital University of Nice were analyzed. These isolates were recovered from randomly consecutive episodes of *S. aureus* infections in patients hospitalized during 2005. These isolates were obtained from various types of clinical samples, comprising blood cultures (28 bacteria); skin infections including chronic ulcers, burns or wounds (83 bacteria); urine samples (41 bacteria); sputum samples (69 bacteria); and various deep-seated soft tissue infections such as subcutaneous or visceral abscesses, spontaneously or post operative soft tissue infections (35 bacteria). For the last group, bacteria were isolated from specimens obtained by guidance radiography needle biopsy or during endoscopic and surgical procedures. All isolates were characterized using routine methods according to each manufacturer's recommendations. All were positive for catalase, DNAse production and mannitol fermentation in Chapman medium, and confirmed to be *S. aureus* by specific 32rapidStaph (BioMérieux, Marcy-l'Etoile, France).

Antibiotic susceptibility determinations

Antimicrobial susceptibility testing was performed on all isolates obtained during the study using the disk diffusion method [22] on Mueller-Hinton medium (Difco Laboratories, Detroit, MI) according to the recommendations of the French Antibiogram Committee [http://www.sfm.asso.fr/nouv/general.php?pa=2]. Antibiotics tested were penicillin G, oxacillin, erythromycin, clindamycin and fusidic acid to focus on epidemiologic profiles.

DNA isolation and PCR-based detection of genes

For *edin* detection, total DNA was isolated from bacterial strains grown overnight at 37°C in BHI medium. Bacteria were lysed in 10 mM TrisHCl pH7.8, 100 mM NaCl, 1mM EDTA, 1% Triton X100. After incubation for 10 minutes at 100°C, DNA was collected and frozen. PCR amplification was used to detect the presence of *edin*-A, B and C using the primers described in Table 1. We have determined optimized thermal cycling conditions for *edin-A* (25 cycles of 30s at 94°C, 45s annealing at 58°C and 1 min elongation at 72°C), *edin-B* (25 cycles of 30s at

95°C, 1 min annealing at 50°C and 1 min elongation at 72°C) and *edin-C* (30 cycles of 30s at 94°C, 45s annealing at 54°C and 1 min elongation at 72°C). For the detection of other virulence genes, total DNA was isolated from bacterial strains grown three hours at 37°C in BHI medium. DNA was subsequently extracted with NucleoSpin Tissue (Macherey-Nagel GmbH, Düren, Germany) according to manufacturer's recommendations. Briefly, bacteria were pelleted by centrifugation at 8,000 ×*g* for 5 min, resuspended in 180µl of lysis buffer with 33µl of proteinase K (20mg/ml) (Invitrogen Life Technologies, Carlsbad, CA) and 3µl of recombinant lysostaphin (3U/µl) (Sigma-Aldrich, St Louis, MI), and incubated for 60 min at 37°C. DNA samples were eluted with 100 µl alkaline elution buffer (BE buffer, NucleoSpin Tissue, Macherey-Nagel). The presence of 30 genes, among the most prevalent virulence-associated genes, was evaluated by PCR as described previously: staphylococcal enterotoxins (*se*) A, B, C, D, E, G, H, I, J, K, and Q, toxic shock syndrome toxin 1 (*tst*-1), exfoliative toxins A, B and D (*etA, etB, etD*), PVL (*luk*S-PV–*luk*F-PV), LukDE leukocidin (*lukE*), nine MSCRAMM (*bbp, cna, ebpS, clfA, clfB, fib, fnbA, fnbB, eno*). The accessory gene regulator (*agr*) allele group was determined by multiplex PCR.

spa sequencing

spa typing was performed as described previously [23], using the spa typing website (http://www.spaserver.ridom.de/) that is developed by Ridom GmbH and curated by SeqNet.org (http://www.SeqNet.org/). Primers are indicated in Table 1.

Statistical analysis

The chi-square or Fisher's exact test for categorical variables was used to compare data as appropriate. A *P* value of less than 0.05 was considered significant.

Results

Detection of *edin* isoforms

S. aureus isolates analyzed in this study were collected at the university hospital of Nice from various infected patients. We designed primers with high sensitivity and specificity in order to detect, by PCR, *edin*-A, B and C alleles in these isolates. This was especially challenging for *edin*-C, which was poorly detected using a previously described pair of primers designed to amplify all *edin* isoforms. This is consistent with the fact that the sequence of *edin-C* has the most substantial sequence variations in regions recognized by these primer sequences (17% and 32% homology with the forward and reverse primers, respectively) (Fig. 1) [19]. As shown in figure 1, the three pairs of primers designed allowed us to amplify specifically a 455 bp DNA fragment for *edin-A* and *B*, and a 320 bp DNA fragment for *edin-C*.

We next analyze the 256 clinical isolates of *S. aureus*. We found that 14 % (36) of these isolates were positive for one of the *edin* alleles. Among these 36 isolates, 90% were positive for *edin-C* and 5% were positive for either *edin-A* or *B*. To confirm the nature of the *edin* isoforms, we performed complete sequence analysis of five *edin-C* encoding genes from randomly selected isolates. We also sequenced *edin-A* and *edin-B* encoding genes. These results confirm the specificity of the new primers used and demonstrate that *edin-C* was more prevalent than other alleles of *edin*.

Detection of genes encoding virulence factors

The 36 isolates positive for *edin* genes were next analyzed for the distribution of major MSCRAMMs, various staphylococcal enterotoxins, exfoliative toxins, the toxic shock syndrome toxin 1 gene *tst-1*, as well as leucotoxin family encoding genes. Among the staphylococcal MSCRAMM genes, *eno, clfA* and *clfB* were detected in all *edin*-positive isolates (Table 2). *bbp, fnbA* and *ebpS* were the less frequently encountered MSCRAMMs among these isolates. However these adhesion factors had no preferential distribution among *S. aureus* isolated from various types of infections. Among the staphylococcal enterotoxins genes the most frequently encountered were *seg*, *sei* and *sea* (Table 2). In addition, 72% of *edin*-positive isolates (26 bacteria) contained a combination of these three genes. One *edin-C* bearing isolate, isolated from a urine sample, had only the *sea* enterotoxin gene. We detected the exfoliative toxin gene *etD* exclusively among the *edin-B* positive isolates. Two *edin*-A

positive isolates carried the staphylococcal exfoliative gene *etB*. We found that 25 *edin-C* positive *S. aureus* (78%) were negative for *etA*, *etB* and *etD* exfoliative toxin encoding genes. We observed that only 22% of the *edin-C* positive *S. aureus* (7 out of 32) had at least one of the two isoforms (*etA* or *etB*) of the exfoliative toxin gene. Five of these seven isolates carried both the *etA* and *etB* genes. No significant association was found between the presence of *tst-1*, exfoliative toxins or leucotoxin family encoded genes and the types of infection. Finally, a large number of *edin*-positive *S. aureus* belonged to the *agr* group 1 (50%, 18 bacteria), and to a lesser extent, to *agr* groups 2, 3 and 4 (17%, 6 bacteria each) (Table 2).

spa-typing

Determination of the *spa* type of 26 *edin*-positive isolates [23] allowed us to exclude the clonal origin of all *edin-C* positive *S. aureus* in our survey. Among them, only six isolates could be classified as ST45 (two isolates), ST30 (two isolates), ST59 or ST26. The other 20 isolates showed a high variability of their *spa* type (Table 3). For 14 isolates, we determined new repeat sequences including unidentified 24-bp repeats thus defining new *spa* types. Among them, bacteria S7926 and S7262, isolated respectively in deep seated soft tissue and sputum sample from unrelated infected patients, presented the same *spa* type t6956 (Table 3). Together these data excluded the clonal origin of all *edin-C* positive *S. aureus* in our survey.

Antibiotic susceptibility profiles

We next investigated whether *edin*-positive isolates were associated with specific antibiotic resistance profiles, such as community-acquired methicillin resistant *S. aureus* ST80 and fusidic acid-resistant impetigo clones [20, 21]. In relation with these studies, we determined the minimum inhibitory concentrations (MICs) of *edin*-positive isolates for the following antibiotics: penicillin G, methicillin, erythromycin, clindamycin and fusidic acid. Results were presented in Table 2. Only one isolate, positive for *edin-C,* showed a methicillin-resistance. Finally, *edin*-positive isolates did not show any specific resistance profile to classical antibiotics used to cure infections by *S. aureus* (Table 2).

Clinical origin of *edin*-positive *S. aureus*

Having shown no link between a high prevalence of *edin-C* within our *S. aureus* isolates and any phenotypic profile or clonal origin, we further analyzed the distribution of *edin*-positive isolates with regard to the infectious sites. We noticed that isolates positive for *edin-C* were recovered at all sites of infection (Table 4). Strikingly, *edin*-positive *S. aureus* were more significantly associated with deep-seated infections of soft tissues than other types of infections (25.7%, Fischer exact test, p=0.03). No significant association was detected between *edin*-positive or *edin*-negative isolates and other types of infections (blood, urine, superficial soft tissue and sputum culture).

Discussion

Our study shows that *edin*-positive *S. aureus* isolates are found in all types of clinical infections included in this study, with a global prevalence of 14%. Moreover, we show that 90% of *edin*-positive isolates are positive for *edin-C*. This is consistent with a previous study performed with specific primers, which also reported a higher prevalence of *edin-C* in clinical isolates of *S. aureus* in Japan [24]. This points for the need of using specific primers to detect each *edin* isoform, especially *edin-C*, given its high prevalence. On the contrary, both studies point for a possible underestimation of the prevalence of *edin-C* in pathogenic *S. aureus* when consensus primers were used. Both the analysis of *spa* type and the distribution of various virulence factors among *edin*-positive *S. aureus* show their high genetic variability. Our results on the distribution of MSCRAMMs are consistent with previous findings [25]. Classically, *edin* and exfoliative toxin encoding genes are associated in specific lineages responsible for skin infections [17, 21, 24]. Interestingly, here we show that *edin-C* is not strictly associated with genes encoding exfoliative toxin of serotypes A/B/D (7 bacteria *edin-C*-positive and *et*-positive, versus 25 bacteria *edin-C*-positive and *et*-negative). The high prevalence of *edin-C*-positive and *et*-negative isolates observed in our study might be explained by the plasticity of the pETB plasmid, which has been previously reported in two different variants [18, 21]. Also, a recent study shows genetic variations in pathogenicity islands encoding EDIN-B [17].

Given that *spa* analysis constitutes a good tool for epidemiological typing of *S. aureus* [26], the use of this method allowed us to exclude the clonal origin of *edin-C* positive isolates. The fact that *edin-C* is plasmid born might explain its presence in isolates of various genetic backgrounds.

S. aureus positive for *edin* are more frequently associated with deep-seated infections of soft tissues. We have previously established that infection of endothelial cells, and other cell types, by EDIN-producing *S. aureus* triggers the formation of transcellular tunnels named macroapertures [12, 13]. Opening of transcellular tunnels in the endothelium suggested that EDIN might favour bacterial extravasation in tissues during bacteremia. In a mouse model of intravascular injection of *S. aureus*, we have observed that EDIN plays no detectable role in the persistence of bacteria in the blood stream [15]. This data is consistent with the absence of a higher prevalence of *edin* positive *S. aureus* recovered from patients associated bacteriemia in this study. In contrast, in this model of mouse infection EDIN toxin promotes formation of infection foci [15]. This suggested that EDIN might enhance the invasive capacity of *S.*

aureus. The hypothesis of a role of EDIN in *S. aureus* infection is also supported by our present findings showing that *S. aureus* associated with deep-seated infections of soft tissues have a higher prevalence of *edin*. However, whether or not *edin*-positive *S. aureus* is preferentially associated with a specific type of deep seated infection remains to be further determined".

Acknowledgments We are grateful to Fernand Girard-Pipau and Claire Poyart for providing various strains of *S. aureus* and Pr Jean-Louis Mege for critical reading of the manuscript.

Our laboratory is supported by an institutional funding from the INSERM, a grant from the Agence Nationale de la Recherche (ANR RPV07055ASA) and the Association pour la Recherche sur le Cancer (ARC 4906).

References

[1] Lowy FD (1998) *Staphylococcus aureus* infections. N Engl J Med 339 (8):520-532

[2] Fournier B, Philpott DJ (2005) Recognition of *Staphylococcus aureus* by the innate immune system. Clin Microbiol Rev 18 (3):521-540

[3] Becker K, Friedrich AW, Lubritz G et al (2003) Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. J Clin Microbiol 41 (4):1434-1439

[4] Boquet P, Lemichez E (2003) Bacterial virulence factors targeting Rho GTPases: parasitism or symbiosis? Trends Cell Biol 13 (5):238-246

[5] Dinges MM, Orwin PM, Schlievert PM (2000) Exotoxins of *Staphylococcus aureus*. Clin Microbiol Rev 13 (1):16-34

[6] Wilde C, Aktories K (2001) The Rho-ADP-ribosylating C3 exoenzyme from *Clostridium botulinum* and related C3-like transferases. Toxicon 39 (11):1647-1660

[7] Wilde C, Vogelsgesang M, Aktories K (2003) Rho-specific *Bacillus cereus* ADP-ribosyltransferase C3cer cloning and characterization. Biochemistry 42 (32):9694-9702

[8] Chardin P, Boquet P, Madaule P et al (1989) The mammalian G protein rhoC is ADP-ribosylated by *Clostridium botulinum* exoenzyme C3 and affects actin microfilaments in Vero cells. EMBO J 8 (4):1087-1092

[9] Aktories K, Barbieri JT (2005) Bacterial cytotoxins: targeting eukaryotic switches. Nat Rev Microbiol 3 (5):397-410

[10] Jaffe AB, Hall A (2005) Rho GTPases: biochemistry and biology. Ann Rev Cell Dev Biol 21:247-269

[11] Visvikis O, Maddugoda MP, Lemichez E (2010) Direct modifications of Rho proteins: deconstructing GTPase regulation. Biol Cell 102 (7):377-389

[12] Boyer L, Doye A, Rolando M et al (2006) Induction of transient macroapertures in endothelial cells through RhoA inhibition by *Staphylococcus aureus* factors. J Cell Biol 173 (5):809-819

[13] Lemichez E, Lecuit M, Nassif X et al (2010) Breaking the wall: targeting of the endothelium by pathogenic bacteria. Nat Rev Microbiol 8 (2):93-104

[14] Rolando M, Munro P, Stefani Cet al (2009) Injection of *Staphylococcus aureus* EDIN by the *Bacillus anthracis* protective antigen machinery induces vascular permeability. Infect Immun 77 (9):3596-3601

[15] Munro P, Benchetrit M, Nahori MA et al (2010) *Staphylococcus aureus* EDIN toxin promotes formation of indection foci in a mouse model of bacteremia. Infect Immun 78 (8):3404-3411

[16] Inoue S, Sugai M, Murooka Y et al (1991) Molecular cloning and sequencing of the epidermal cell differentiation inhibitor gene from *Staphylococcus aureus*. Biochem biophys Research Com 174 (2):459-464

[17] Franke GC, Bockenholt A, Sugai M et al (2009) Epidemiology, variable genetic organisation and regulation of the EDIN-B toxin in *Staphylococcus aureus* from bacteraemic patients. Microbiology 156 (3):860-872.

[18] Yamaguchi T, Hayashi T, Takami H et al (2001) Complete nucleotide sequence of a *Staphylococcus aureus* exfoliative toxin B plasmid and identification of a novel ADP-ribosyltransferase, EDIN-C. Infect Immun 69 (12):7760-7771

[19] Czech A, Yamaguchi T, Bader L et al (2001) Prevalence of Rho-inactivating epidermal cell differentiation inhibitor toxins in clinical *Staphylococcus aureus* isolates. J Infect Dis 184 (6):785-788

[20] Ben Nejma M, Mastouri M, Bel Hadj Jrad B et al (2008) Characterization of ST80 Panton-Valentine leukocidin-positive community-acquired methicillin-resistant *Staphylococcus aureu*s clone in Tunisia. Diagn Microbiol Infectious Dis In press [Epub ahaed of print]

[21] O'Neill AJ, Larsen AR, Skov R, Henriksen AS, Chopra I (2007) Characterization of the epidemic European fusidic acid-resistant impetigo clone of *Staphylococcus aureus*. J Clin Microbiol 45 (5):1505-1510

[22] Bauer AW, Kirby WM, Sherris JC et al (1966) Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 45 (4):493-496

[23] Harmsen D, Claus H, Witte W et al (2003) Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for spa repeat determination and database management. J Clin Microbiol 41 (12):5442-5448

[24] Yamaguchi T, Yokota Y, Terajima J et al (2002) Clonal association of *Staphylococcus aureus* causing bullous impetigo and the emergence of new methicillin-resistant clonal groups in Kansai district in Japan. J Infect Dis 185 (10):1511-1516

[25] Tristan A, Ying L, Bes M et al (2003) Use of multiplex PCR to identify *Staphylococcus aureus* adhesins involved in human hematogenous infections. J Clin Microbiol 41 (9):4465-4467

[26] Petersson AC, Olsson-Liljequist B, Miorner H, et al (2010) Evaluating the usefulness of spa typing, in comparison with pulsed-field gel electrophoresis, for epidemiological typing of methicillin-resistant *Staphylococcus aureus* in a low-prevalence region in Sweden 2000-2004. Clin Microbiol Infect 16 (5):456-462

[27] Holtfreter S, Bauer K, Thomas D et al (2004) egc-Encoded superantigens from *Staphylococcus aureus* are neutralized by human sera much less efficiently than are classical staphylococcal enterotoxins or toxic shock syndrome toxin. Infect Immun 72 (7):4061-4071

[28] Johnson WM, Tyler SD, Ewan EP et al (1991) Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. J Clin Microbiol 29 (3):426-430

[29] Jarraud S, Mougel C, Thioulouse J et al (2002) Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. Infect Immun 70 (2):631-641

[30] Lina G, Boutite F, Tristan A et al (2003) Bacterial competition for human nasal cavity colonization: role of Staphylococcal agr alleles. Appl Environmental Microbiol 69 (1):18-23

Figure legend

Fig. 1 Characterization of the three *edin* alleles. A) PCR amplification of *edin-A*, *edin-B* and *edin-C* from *S. aureus* strains S25*edin-A(*+)[15], S7256*edin-B(*+) and S7475*edin-C(*+) (this study), respectively, using specific oligonucleotides *edinA*, *edinB*, *edinC* (Table 1) and the previously described *edin* oligonucleotides referred as *edinX* [19]. B) Sequence alignments of *edin-A*, *edin-B* and *edin-C* showing sequence homologies and localization of each oligonucleotide (underlined: *edinX*; highlighted: *edinA*, *edinB* and *edinC*).

1 Table 1: Oligonucleotides primers used in this study

2

Gene	Primer sequences	Size (kb)	References
*edin*A	Sense 5'-GGAGATATTAATAAGCTAGATTC-3'	455	This study
	Antisense 5'-ATTTTCTTTTTATCATTTGACAATTCT-3'		
*edin*B	Sense 5'-GGTGACGTGAACAAATTATCCGA-3'	455	This study
	Antisense 5'-ATCTTTCTTTTGTTATCAGAAAGTTTA-3'		
*edin*C	Sense 5'-CGCCATTAAGGTCTAGTCAAGG-3'	320	This study
	Antisense 5'-TAGGTCTTCCAGCTAATGCAGC-3'		
bbp	Sense 5'-AACTACATCTAGTACTCAACAACAG-3'	575	[25]
	Antisense 5'-ATGTGCTTGAATAACACCATCATCT-3'		
cna	Sense 5'-GTCAAGCAGTTATTAACACCAGAC-3'	423	[25]
	Antisense 5'-AATCAGTAATTGCACTTTGTCCACTG-3'		
ebpS	Sense 5'-CATCCAGAACCAATCGAAGAC-3'	186	[25]
	Antisense 5'-CTTAACAGTTACATCATCATGTTTATCTTTG-3'		
fnbA	Sense 5'-GTGAAGTTTTAGAAGGTGGAAAGATTAG -3'	643	[25]
	Antisense 5'-GCTCTTGTAAGACCATTTTTCTTCAC-3'		
fnbB	Sense 5'-GTAACAGCTAATGGTCGAATTGATACT-3'	524	[25]
	Antisense 5'-CAAGTTCGATAGGAGTACTATGTTC-3'		
fib	Sense 5'-CTACAACTACAATTGCCGTCAACAG-3'	404	[25]
	Antisense 5'-GCTCTTGTAAGACCATTTTCTTCAC-3'		
clfA	Sense 5'-ATTGGCGTGGCTTCAGTGCT-3'	292	[25]
	Antisense 5'-CGTTTCTTCCGTAGTTGCATTTG-3'		
clfB	Sense 5'-ACATCAGTAATAGTAGGGGGCAAC-3'	205	[25]
	Antisense 5'-TTCGCACTGTTTGTGTTTGCAC-3'		
eno	Sense 5'- ACGTGCAGCAGCTGACT-3'	302	[25]
	Antisense 5'- CAACAGCATYCTTCAGTACCTTC-3'		
sea	Sense 5'-GCAGGGAACAGCTTTAGGC-3'	520	[27]
	Antisense 5'-GTTCTGTAGAAGTATGAAACACG-3'		
seb	Sense 5'-ATGTAATTTTGATATTCGCAGTG-3'	683	[27]
	Antisense 5'-TGCAGGCATCATATCATACCA-3'		
sec	Sense 5'-CTTGTATGTATGGAGGAATAACAA-3'	283	[27]

	Antisense 5’-TGCAGGCATCATATCATACCA-3’		
sed	Sense 5’-GTGGTGAAATAGATAGGACTGC-3’	384	[27]
	Antisense 5’-ATATGAAGGTGCTCTGTGG-3’		
see	Sense 5’-TACCAATTAACTTGTGGATAGAC-3’	170	[27]
	Antisense 5’-CTCTTTGCACCTTACCGC-3’		
seg	Sense 5’-CGTCTCCACCTGTTGAAGG-3’	327	[27]
	Antisense 5’-CCAAGTGATTGTCTATTGTCG-3’		
seh	Sense 5’-CAACTGCTGATTTAGCTCAG-3’	360	[27]
	Antisense 5’-GTCGAATGAGTAATCTCTAGG-3’		
sei	Sense 5’-CAACTCGAATTTTCAACAGGTAC-3’	465	[27]
	Antisense 5’- CAGGCAGTCCATCTCCTG-3’		
sej	Sense 5’- CATCAGAACTGTTGTTCCGCTAG-3’	142	[27]
	Antisense 5’- CTGAATTTTACCATCAAAGGTAC-3’		
sek	Sense 5’- ATGGCGGAGTCACAGCTACT-3’	197	[27]
	Antisense 5’-TGCCGTTATGTCCATAAATGTT-3’		
seq	Sense 5’-GAACCTGAAAAGCTTCAAGGA-3’	209	[27]
	Antisense 5’-ATTCGCCAACGTAATTCCAC-3’		
eta	Sense 5’-CTAGTGCATTTGTTATTCAA-3’	119	[28]
	Antisense 5’-TGCATTGACACCATAGTACT-3’		
etb	Sense 5’-ACGGCTATATACATTCAATT-3’	200	[28]
	Antisense 5’-TCCATCGATAATATACCTAA-3’		
etd	Sense 5'-ATGACTAAAAATATATTAAAAAGTT-3’	846	This study
	Antisense 5‘-CTAATGAGACTGTAATTCAGC-3’		
lukPV	Sense 5’-ATCATTAGGTAAAATGTCTGGACATGATCCA-3’	433	[29]
	Antisense 5’-GCATCAASTGTATTGGATAGCAAAAGC-3’		
lukE	Sense 5’-TGAAAAAGGTTCAAAGTTGATACGAG-3’	269	[29]
	Antisense 5’-TGTATTCGATAGCAAAAGCAGTGCA-3’		
tst-1	Sense 5’-GCTTGCGACAACTGCTACAG-3’	559	[27]
	Antisense 5’-TGGATCCGTCATTCATTGTTAA-3’		
agr1	Sense 5’-ATGCACATGG TGCACATGC-3’	439	[30]
	Antisense 5’-GTCACAAGTACTATAAGCTGCGAT-3’		
agr2	Sense 5’-ATGCACATGG TGCACATGC-3’	572	[30]
	Antisense 5’-TATTACTAATTGAAAAGTGC CATAGC-3’		

agr3	Sense 5’-ATGCACATGG TGCACATGC-3’	321	[30]
	Antisense 5’-GTAATGTAATAGCTTGTATAATAATACCCAG-3’		
agr4	Sense 5’-ATGCACATGG TGCACATGC-3’	657	[30]
	Antisense 5’-CGATAATGCCGTAATACCCG-3’		
spa	Sense5’-TGTAAAACGACGGCCAGTTAAAGACGATCCTTCGGTGAGC-3’		[23]
	Antisense5’-CAGGAAACAGCTATGACCCAGCAGTAGTGCCGTTTGCTT-3’		

1

Table 2: Virulence profile and antibiotic susceptibility of clinical *edin*-positive *S. aureus* isolates.

	blood (n=2)		superficial soft tissue (n=11)		urine sample (n=4)		sputum sample (n=10)		deep-seated soft tissue (n=9)		total (n=36)	
	n	%	n	%	n	%	n	%	n	%	n	%
Virulence profile												
MSCRAMMs												
bbp	0	0	3	27	3	75	3	30	6	67	15	42
cna	1	50	9	82	4	100	10	100	8	89	32	89
ebpS	0	0	5	45	1	25	6	60	6	67	18	50
fnbA	1	50	7	64	1	25	5	50	5	56	19	53
fnbB	1	50	11	100	4	100	7	70	8	89	31	86
fib	2	100	10	91	4	100	7	70	9	100	32	89
clfA	2	100	11	100	4	100	10	100	9	100	36	100
clfB	2	100	11	100	4	100	10	100	9	100	36	100
eno	2	100	11	100	4	100	10	100	9	100	36	100
SEs												
sea	1	50	10	91	3	75	9	90	9	100	32	89
seb	1	50	7	64	2	50	8	80	6	67	24	67
sec	1	50	7	64	2	50	10	100	6	67	26	72
sed	0	0	8	73	3	75	6	60	6	67	23	64
see	0	0	0	0	0	0	0	0	0	0	0	0
sek	1	50	5	45	1	25	7	70	2	22	16	44
seq	1	50	3	27	1	25	5	50	5	56	15	42
seg	2	100	10	91	2	50	10	100	9	100	33	92
seh	0	0	0	0	0	0	0	0	1	11	1	3
sei	2	100	11	100	2	50	9	90	8	89	32	89
sej	0	0	1	9	0	0	2	20	1	11	4	11
tst-1	2	100	6	55	1	25	7	70	5	56	21	58
etA	0	0	2	18	0	0	2	20	2	22	6	17
etB	1[&]	50	4[$]	36	0	0	2	20	1	11	8	22
etD	0	0	0	0	1*	25	1*	10	0	0	2	6
lukPV	0	0	1	9	1	25	0	0	3	33	5	14
lukE	2	100	8	73	3	75	5	50	4	44	24	67
agr group												
agr1	1	50	3	27	3	75	6	60	5	56	18	50
agr2	1	50	3	27	1	25	1	10	0	0	6	17
agr3	0	0	3	27	0	0	0	0	3	33	6	17
agr4	0	0	2	18	0	0	3	30	1	11	6	17
antibiotic resistance profile												
penicillin G	1	50	5	45	2	50	8	80	6	67	22	61
Methicillin	0	0	0	0	0	0	0	0	1	11	1	3
Erythromycin	0	0	1	9	0	0	1	10	1	11	3	8
Clindamycin	0	0	0	0	0	0	0	0	1	11	1	3
Fusidic acid	0	0	0	0	1¤	25	0	0	1¤	11	2	6

* *Edin B* positive strains
[$] *Edin A* positive for one strain
[&] *EdinA* positive strain
¤ Increase in fusidic acid MIC, classified as intermediary sensibility

Table 3 : *spa*-type of 36 *edin*-positive *Staphylococcus aureus* isolates.

Strains	EDIN type	*Spa*-type or repeat sequences	MLST
Blood			
S25	A	t6403	-
S7232	C	NT[$]	-
Deep-seated soft tissue			
S7272	C	t6953	-
S7404	C	t6649	-
S7408	C	t6484	-
S7466	C	t012	ST-30
S7595	C	NT	-
S7600	C	t6677	-
S7926	C	t6956	-
S8028*	C	NT	-
S8087	C	t6481	-
Sputum sample			
S7225	C	t2726	-
S7259	C	t2088	-
S7262	C	t6956	-
S7413	C	t6483	-
S7535	C	t2647	-
S7634	C	t6954	-
S7649	B	NT	-
S7920	C	NT	-
S7965	C	NT	-
S8100	C	t015	ST-45
Superficial soft tissue			
S7181	C	t6650	-
S7183	C	NT	-
S7436	C	t6957	-
S7475	C	t6480	-
S7526	C	t137	-

S7539	C	NT	-
S7569	A	t031	ST-45
S7599	C	t6482	-
S7932	C	NT	-
S7938	C	t620	-
S7977	C	NT	-
Urine sample			
S7256	B	t078	ST-26
S7322	C	t012	ST-30
S7906	C	t645	-
S7946	C	t216	ST-59

* MRSA bacteria

$ Not typable

Table 4: Presence of *edin* genes in 256 *Staphylococcus aureus* isolates associated with various clinical syndromes.

Source of isolates (N)	**Number of *edin* isolates (%)**	***edin*-isoforms**		
		*edin*A	*edinB*	*edinC*
Blood (28)	2 (7.1)	1	0	1
Urine (41)	4 (9.8)	0	1	3
Superficial soft tissue (83)	11 (13.3)	1	0	10
Deep-seated soft tissue (35)	9 (25.7)*	0	0	9
Sputum (69)	10 (14.5)	0	1	9
Total (256)	**36 (14)**	**2 (5)**	**2 (5)**	**32 (90)**

* p<0,05 (Fisher's exact test)

High prevalence of *edin-C* encoding RhoA-targeting toxin in clinical strains of *Staphylococcus aureus*

Patrick Munro[1], René Clément[1], Jean-Philippe Lavigne[4,5], Céline Pulcini[2,3], Emmanuel Lemichez[1] and Luce Landraud[1,6*]

Running title: EDIN exotoxins in *S. aureus* infections

1 INSERM, U895, C3M, toxines microbiennes dans la relation hôte pathogènes, Université de Nice-Sophia-Antipolis, UFR Médecine, IFR50, Nice, F-06204, France

2 Université de Nice-Sophia-Antipolis, UFR Médecine, IFR50, Nice, F-06204, France

3 Service d'Infectiologie, Hôpital l'Archet 1, Route Saint Antoine de Ginestière, BP 3079, 06202 Nice Cedex 3, France

4 INSERM, Espri 26, Université Montpellier 1, UFR de Médecine, Nîmes, France

5 Laboratoire de Bactériologie, CHU Caremeau, Nîmes, France

6 Laboratoire de Bactériologie, CHU de Nice, Hôpital l'Archet, Nice, France.

*** Corresponding author:**

Luce Landraud, INSERM, U895, C3M, toxines microbiennes dans la relation hôte pathogènes, Université de Nice-Sophia-Antipolis, UFR Médecine, IFR50, Nice, F-06204, France, and Laboratoire de Bactériologie, CHU de Nice, Hôpital l'Archet, Nice, France.

Telephone: 00 33 4 89 06 42 61

Fax: 00 33 4 89 06 42 60

Mail: landraud.l@chu-nice.fr

Abstract word count: 173

Abstract *Staphylococcus aureus*, a major causative agent of human infection produces a large array of virulence factor including various toxins. Among them, the host RhoA GTPase targeting EDIN toxins are considered as potential virulence factors. Using the polymerase chain reaction, we analyzed the virulence profile of 256 *S. aureus* strains isolated from various clinical sites of infections. We developed specific primers to detect the three isoforms of *edin* encoding genes. We found a prevalence of 14% (36 strains) of *edin* encoding genes among these clinical strains. Strikingly, we found that 90% of all *edin*-bearing *S. aureus* strains carried the type-C allele. Both the *spa* types and the profile of virulence factors of these *edin*-positive strains are highly variable. Notably, we show for the first time that *edin*-C positive strains were more frequently recovered from deep-seated infections than other types of infections. Our present work thus strongly suggests that presence of *edin-C* is a risk factor of *S. aureus* dissemination in tissues and thus represents a predictive marker for a pejorative evolution of staphylococcal infections.

Keywords

Staphylococcus aureus, EDIN, toxin, ADP-ribosyltransferase, virulence factors, Rho GTPases.

Introduction

Staphylococcus aureus is a common bacterium, which is responsible for a unique variety of infections [1]. Development of pejorative forms of staphylococcal infections involves the combined action of numerous bacterial virulence factors, which corrupt host responses [2]. Bacterial virulence factors include specific adhesins, collectively referred as Microbial Surface Components Recognizing endothelial cell Adhesive Matrix Molecules (MSCRAMMs) and a large variety of toxins, such as the exfoliative toxins (ETs), hemolysin, leukocidin, enterotoxins and EDINs (epidermal cell differentiation inhibitors) [3-6].

EDINs belong to the family of *Clostridium botulinum* C3 exoenzyme [6, 7]. They are members of a group of major bacterial virulence factors targeting host Rho GTPases [4, 6-9]. Rho proteins control essential cellular processes such as cell polarity, movement and phagocytosis, as well as cohesion of intercellular junctions [6, 10, 11]. Recent findings suggest that EDINs might favor bacterial dissemination in tissues, by a haematogenous route, through induction of large transcellular tunnels in endothelial cells named macroapertures [12-14]. Indeed, recent data show that *S. aureus* EDIN toxin promotes formation of infection foci in a mouse model of bacteremia [15]. To date, three isoforms of EDIN have been characterized. These comprise the first discovered EDIN isoform (EDIN-A), isolated from the E-1 strain of *S. aureus* [16], as well as EDIN-B [6, 17] and EDIN-C [18]. The chromosomal gene encoding *edin-B* is located within a pathogenicity island frequently associated with the *etD* gene encoding the exfoliative toxin ET-D [17]. EDIN-C is encoded by the pETB plasmid, which also carries genes encoding ET-B and conferring cadmium resistance [18].

A first epidemiological survey, involving staphylococcal strains isolated from patients hospitalized for various infectious diseases demonstrated a higher prevalence of *edin*-encoding genes in this group compared to nasal strains isolated from healthy students [19]. Another study shows that *edin-B* is present in 7% of bacteriemic *S. aureus* strains [17]. However, most other epidemiological data on *edin* are based on surveys focused on exfoliative toxins or PVL rather than EDIN toxin itself. For example, a genetic association between *etD* and *edin-B* was detected in the emerging ST80 clone Panton-Valentine Leukocidin (PVL)-positive and community-acquired (CA) methicillin-resistant *S. aureus* (MRSA) [20]. This clone is spreading throughout France and Tunisia and is most frequently associated with infections of the skin and soft tissues. Also, two-thirds of the strains belonging to the emerging ST123 epidemic European fusidic acid-resistant impetigo clone

(EEFIC) were positive for *etB* and sequence analysis of pETB2 (a close homolog of pETB) in one of these strains suggested that it also bears *edin-C* [21].

In this study, we have developed a PCR-based method, to detect EDIN isoforms specifically. We demonstrate that 90% of all *edin*-bearing *S. aureus* strains carry the type-C allele. We also show that these strains are more significantly associated with deep-seated soft tissue infections than other types of infections (Fisher's exact test, $p=0.03$).

Materials and methods

S. aureus isolates

A total of 256 strains of *S. aureus* were retrospectively collected from patients hospitalized at the university hospital of Nice during 2005. These strains were obtained from various types of clinical samples, comprising blood cultures (28 strains); skin infections including chronic ulcers, burns or wounds (83 strains); urine samples (41 strains); sputum samples (69 strains); and various deep-seated soft tissue infections such as subcutaneous or visceral abscesses, spontaneously or post operative soft tissue infections (35 strains). All isolates were characterized using routine methods according to each manufacturer's recommendations. All were positive for catalase, DNAse production and mannitol fermentation in Chapman medium, and confirmed to be *S. aureus* by specific 32rapidStaph (BioMérieux, Marcy-l'Etoile, France).

Antibiotic susceptibility determinations

Antimicrobial susceptibility testing was performed on all isolates obtained during the study using the disk diffusion method [22] on Mueller-Hinton medium (Difco Laboratories, Detroit, MI) according to the recommendations of the French Antibiogram Committee [http://www.sfm.asso.fr/nouv/general.php?pa=2]. Antibiotics tested were penicillin G, oxacillin, erythromycin, clindamycin and fusidic acid to focus on epidemiologic profiles.

DNA isolation and PCR-based detection of genes

For *edin* detection, total DNA was isolated from bacterial strains grown overnight at 37°C in BHI medium. Bacteria were lysed in 10 mM TrisHCl pH7.8, 100 mM NaCl, 1mM EDTA, 1% Triton X100. After incubation for 10 minutes at 100°C, DNA was collected and frozen. PCR amplification was used to detect the presence of *edin*-A, B and C using the primers described in Table 1. We have determined optimized thermal cycling conditions for *edin-A* (25 cycles of 30s at 94°C, 45s annealing at 58°C and 1 min elongation at 72°C), *edin-B* (25 cycles of 30s at 95°C, 1 min annealing at 50°C and 1 min elongation at 72°C) and *edin-C* (30 cycles of 30s at 94°C, 45s annealing at 54°C and 1 min elongation at 72°C). For the detection of other virulence genes, total DNA was isolated from bacterial strains grown three hours at 37°C in

BHI medium. DNA was subsequently extracted with NucleoSpin Tissue (Macherey-Nagel GmbH, Düren, Germany) according to manufacturer's recommendations. Briefly, bacteria were pelleted by centrifugation at 8,000 ×*g* for 5 min, resuspended in 180µl of lysis buffer with 33µl of proteinase K (20mg/ml) (Invitrogen Life Technologies, Carlsbad, CA) and 3µl of recombinant lysostaphin (3U/µl) (Sigma-Aldrich, St Louis, MI), and incubated for 60 min at 37°C. DNA samples were eluted with 100 µl alkaline elution buffer (BE buffer, NucleoSpin Tissue, Macherey-Nagel). The presence of 30 genes, among the most prevalent virulence-associated genes, was evaluated by PCR as described previously: staphylococcal enterotoxins (*se*) A, B, C, D, E, G, H, I, J, K, and Q, toxic shock syndrome toxin 1 (*tst*-1), exfoliative toxins A, B and D (*etA, etB, etD*), PVL (*luk*S-PV–*luk*F-PV), LukDE leukocidin (*lukE*), nine MSCRAMM (*bbp, cna, ebpS, clfA, clfB, fib, fnbA, fnbB, eno*). The accessory gene regulator (*agr*) allele group was determined by multiplex PCR.

spa sequencing

spa typing was performed as described previously [23], using the spa typing website (http://www.spaserver.ridom.de/) that is developed by Ridom GmbH and curated by SeqNet.org (http://www.SeqNet.org/). Primers are indicated in Table 1.

Statistical analysis

The chi-square or Fisher's exact test for categorical variables was used to compare data as appropriate. A *P* value of less than 0.05 was considered significant.

Results

Detection of *edin* isoforms

S. aureus isolates analyzed in this study were collected at the university hospital of Nice from various infected patients. We designed primers with high sensitivity and specificity in order to detect, by PCR, *edin*-A, B and C alleles in these strains. This was especially challenging for *edin*-C, which was poorly detected using a previously described pair of primers designed to amplify all *edin* isoforms. This is consistent with the fact that the sequence of *edin-C* has the most substantial sequence variations in regions recognized by these primer sequences (17% and 32% homology with the forward and reverse primers, respectively) (Fig. 1) [19]. As shown in figure 1, the three pairs of primers designed allowed us to amplify specifically a 455 bp DNA fragment for *edin-A* and *B*, and a 320 bp DNA fragment for *edin-C*.

We next analyze the 256 clinical strains of *S. aureus*. We found that 14 % (36) of these strains were positive for one of the *edin* alleles. Among these 36 strains, 90% were positive for *edin-C* and 5% were positive for either *edin-A* or *B*. To confirm the nature of the *edin* isoforms, we performed complete sequence analysis of five *edin-C* encoding genes from randomly selected strains. We also sequenced *edin-A* and *edin-B* encoding genes. These results confirm the specificity of the new primers used and unravel that *edin-C* was more prevalent than other alleles of *edin*.

Detection of genes encoding virulence factors

The 36 strains positive for *edin* genes were next analyzed for the distribution of major MSCRAMMs, various staphylococcal enterotoxins, exfoliative toxins, the toxic shock syndrome toxin 1 gene *tst-1*, as well as leucotoxin family encoding genes. Among the staphylococcal MSCRAMM genes, *eno, clfA* and *clfB* were detected in all *edin*-positive strains (Table 2). *bbp, fnbA* and *ebpS* were the less frequently encountered MSCRAMMs among these strains. However these adhesion factors had no preferential distribution among *S. aureus* isolated from various types of infections. Among the staphylococcal enterotoxins genes the most frequently encountered were *seg*, *sei* and *sea* (Table 2). In addition, 72% of *edin*-positive strains (26 strains) presented a combination of these three genes. One *edin-C* bearing strain, isolated from a urine sample, had only the *sea* enterotoxin gene. We detected the exfoliative toxin gene *etD* exclusively among the *edin-B* positive strains. Two *edin*-A

positive strains carried the staphylococcal exfoliative gene *etB*. We found that 25 *edin-C* positive *S. aureus* (78%) were negative for *etA*, *etB* and *etD* exfoliative toxin encoding genes. We observed that only 22% of the *edin-C* positive *S. aureus* (7 out of 32) had at least one of the two isoforms (*etA* or *etB*) of the exfoliative toxin gene. Five of these seven strains carried both the *etA* and *etB* genes. Finally, a large number of *edin*-positive *S. aureus* belonged to the *agr* group 1 (50%, 18 strains), and to a lesser extent, to *agr* groups 2, 3 and 4 (17%, 6 strains each) (Table 2).

spa-typing

Determination of the *spa* type of 26 *edin*-positive strains [23] allowed us to exclude the clonal origin of all *edin-C* positive *S. aureus* in our survey. Among them, only six strains could be classified as ST45 (two isolates), ST30 (two isolates), ST59 or ST26. The other 20 strains showed a high variability of their *spa* type (Table 3). For 14 strains, we determined new repeat successions including unidentified 24-bp repeats thus defining new *spa* types. Among them, strains S7926 and S7262, isolated respectively in deep seated soft tissue and sputum sample from unrelated infected patients, presented the same *spa* type t6956 (Table 3). Together these data excluded the clonal origin of all *edin-C* positive *S. aureus* in our survey.

Antibiotic susceptibility profiles

We next investigated whether *edin*-positive strains were associated with specific antibiotic resistance profiles, such as community-acquired methicillin resistant *S. aureus* ST80 and fusidic acid-resistant impetigo clones [20, 21]. In relation with these studies, we determined the minimum inhibitory concentrations (MICs) of *edin*-positive strains for the following antibiotics: penicillin G, methicillin, erythromycin, clindamycin and fusidic acid. Fourteen *edin*-positive strains were susceptible to all tested antimicrobial molecules (41.6 %), except fusidic acid. Indeed, one of 14 isolates tested presented only an increase in fusidic acid MIC, classified as intermediary sensibility. Twenty two isolates were resistant to penicillin G. Among them, 19 were susceptible to all other antimicrobial molecules tested (83%) and two strains also presented erythromycin resistance (8.7%). Only one strain, positive for *edin-C*, showed a methicillin-resistance associated to additional resistance, i.e. erythromycin and clindamycin as well as an increase of MIC to fusidic acid. This strain was negative for *etA*

and *etB*. Finally, *edin*-positive strains did not show any specific resistance profile to classical antibiotics used to cure infections by *S. aureus* (Table 2).

Clinical origin of *edin*-positive *S. aureus*

Having shown no link between a high prevalence of *edin-C* within our *S. aureus* strains and any phenotypic profile or clonal origin, we further analyzed the distribution of *edin*-positive strains with regard to the infectious sites. We noticed that strains positive for *edin-C* were recovered at all sites of infection (Table 4). Strikingly, *edin*-positive *S. aureus* were more significantly associated with deep-seated infections of soft tissues than other types of infections (25.7%, Fischer exact test, p=0.03). No significant association was detected between *edin*-positive or *edin*-negative strains and other types of infections (blood, urine, superficial soft tissue and sputum culture).

Discussion

Our study shows that *edin*-positive *S. aureus* strains are found in all types of clinical infections included in this study, with a global prevalence of 14%. Moreover, we show that 90% of *edin*-positive strains are positive for *edin-C*. This is consistent with a previous study performed with specific primers, which also reported a higher prevalence of *edin-C* in clinical strains of *S. aureus* in Japan [24]. Both studies point for a possible underestimation of the prevalence of *edin-C* in pathogenic *S. aureus*. This points for the need of using specific primers to detect each *edin* isoform, especially *edin-C*, given its high prevalence.

Both the analysis of *spa* type and the distribution of various virulence factors among *edin*-positive *S. aureus* show their high genetic variability. Our results on the distribution of MSCRAMMs are consistent with previous findings [25]. Classically, *edin* and exfoliative toxin encoding genes are associated in specific lineages responsible for skin infections [17, 21, 24]. Interestingly, here we show that *edin-C* is not strictly associated with genes encoding exfoliative toxin of serotypes A/B/D (7 strains *edin-C*-positive and *et*-positive, versus 25 strains *edin-C*-positive and *et*-negative). The high prevalence of *edin-C*-positive and *et*-negative strains observed in our study might be explained by the plasticity of the pETB plasmid, which has been previously reported in two different variants [18, 21]. Also, a recent study shows genetic variations in pathogenicity islands encoding EDIN-B [17].

Given that *spa* analysis constitutes a good tool for epidemiological typing of *S. aureus* [26], the use of this method allowed us to exclude the clonal origin of *edin-C* positive strains. The fact that *edin-C* is plasmid born might explain its presence in strains of various genetic backgrounds.

S. aureus positive for *edin* are more frequently associated with deep-seated infections of soft tissues. Infection of endothelial cells, and other cell types, by EDIN-producing *S. aureus* triggers the formation of transcellular tunnels named macroapertures [12, 13]. Moreover, we recently reported that *S. aureus* EDIN toxin promotes formation of infection foci in a mouse model of bacteremia [15]. Together with the present study, this suggests that EDIN might enhance the invasive capacity of *S. aureus*. However, whether or not *edin*-positive *S. aureus* is preferentially associated with a specific type of deep seated infection remains to be further determined.

Acknowledgments We are grateful to Fernand Girard-Pipau and Claire Poyart for providing various strains of *S. aureus* and Pr Jean-Louis Mege for critical reading of the manuscript.

Our laboratory is supported by an institutional funding from the INSERM, a grant from the Agence Nationale de la Recherche (ANR RPV07055ASA) and the Association pour la Recherche sur le Cancer (ARC 4906).

References

[1] Lowy FD (1998) *Staphylococcus aureus* infections. N Engl J Med 339 (8):520-532

[2] Fournier B, Philpott DJ (2005) Recognition of *Staphylococcus aureus* by the innate immune system. Clin Microbiol Rev 18 (3):521-540

[3] Becker K, Friedrich AW, Lubritz G et al (2003) Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. J Clin Microbiol 41 (4):1434-1439

[4] Boquet P, Lemichez E (2003) Bacterial virulence factors targeting Rho GTPases: parasitism or symbiosis? Trends Cell Biol 13 (5):238-246

[5] Dinges MM, Orwin PM, Schlievert PM (2000) Exotoxins of *Staphylococcus aureus*. Clin Microbiol Rev 13 (1):16-34

[6] Wilde C, Aktories K (2001) The Rho-ADP-ribosylating C3 exoenzyme from *Clostridium botulinum* and related C3-like transferases. Toxicon 39 (11):1647-1660

[7] Wilde C, Vogelsgesang M, Aktories K (2003) Rho-specific *Bacillus cereus* ADP-ribosyltransferase C3cer cloning and characterization. Biochemistry 42 (32):9694-9702

[8] Chardin P, Boquet P, Madaule P et al (1989) The mammalian G protein rhoC is ADP-ribosylated by *Clostridium botulinum* exoenzyme C3 and affects actin microfilaments in Vero cells. EMBO J 8 (4):1087-1092

[9] Aktories K, Barbieri JT (2005) Bacterial cytotoxins: targeting eukaryotic switches. Nat Rev Microbiol 3 (5):397-410

[10] Jaffe AB, Hall A (2005) Rho GTPases: biochemistry and biology. Ann Rev Cell Dev Biol 21:247-269

[11] Visvikis O, Maddugoda MP, Lemichez E (2010) Direct modifications of Rho proteins: deconstructing GTPase regulation. Biol Cell 102 (7):377-389

[12] Boyer L, Doye A, Rolando M et al (2006) Induction of transient macroapertures in endothelial cells through RhoA inhibition by *Staphylococcus aureus* factors. J Cell Biol 173 (5):809-819

[13] Lemichez E, Lecuit M, Nassif X et al (2010) Breaking the wall: targeting of the endothelium by pathogenic bacteria. Nat Rev Microbiol 8 (2):93-104

[14] Rolando M, Munro P, Stefani Cet al (2009) Injection of *Staphylococcus aureus* EDIN by the *Bacillus anthracis* protective antigen machinery induces vascular permeability. Infect Immun 77 (9):3596-3601

[15] Munro P, Benchetrit M, Nahori MA et al (2010) *Staphylococcus aureus* EDIN toxin promotes formation of indection foci in a mouse model of bacteremia. Infect Immun 78 (8):3404-3411

[16] Inoue S, Sugai M, Murooka Y et al (1991) Molecular cloning and sequencing of the epidermal cell differentiation inhibitor gene from *Staphylococcus aureus*. Biochem biophys Research Com 174 (2):459-464

[17] Franke GC, Bockenholt A, Sugai M et al (2009) Epidemiology, variable genetic organisation and regulation of the EDIN-B toxin in *Staphylococcus aureus* from bacteraemic patients. Microbiology 156 (3):860-872.

[18] Yamaguchi T, Hayashi T, Takami H et al (2001) Complete nucleotide sequence of a *Staphylococcus aureus* exfoliative toxin B plasmid and identification of a novel ADP-ribosyltransferase, EDIN-C. Infect Immun 69 (12):7760-7771

[19] Czech A, Yamaguchi T, Bader L et al (2001) Prevalence of Rho-inactivating epidermal cell differentiation inhibitor toxins in clinical *Staphylococcus aureus* isolates. J Infect Dis 184 (6):785-788

[20] Ben Nejma M, Mastouri M, Bel Hadj Jrad B et al (2008) Characterization of ST80 Panton-Valentine leukocidin-positive community-acquired methicillin-resistant *Staphylococcus aureu*s clone in Tunisia. Diagn Microbiol Infectious Dis In press [Epub ahaed of print]

[21] O'Neill AJ, Larsen AR, Skov R, Henriksen AS, Chopra I (2007) Characterization of the epidemic European fusidic acid-resistant impetigo clone of *Staphylococcus aureus*. J Clin Microbiol 45 (5):1505-1510

[22] Bauer AW, Kirby WM, Sherris JC et al (1966) Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 45 (4):493-496

[23] Harmsen D, Claus H, Witte W et al (2003) Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for spa repeat determination and database management. J Clin Microbiol 41 (12):5442-5448

[24] Yamaguchi T, Yokota Y, Terajima J et al (2002) Clonal association of *Staphylococcus aureus* causing bullous impetigo and the emergence of new methicillin-resistant clonal groups in Kansai district in Japan. J Infect Dis 185 (10):1511-1516

[25] Tristan A, Ying L, Bes M et al (2003) Use of multiplex PCR to identify *Staphylococcus aureus* adhesins involved in human hematogenous infections. J Clin Microbiol 41 (9):4465-4467

[26] Petersson AC, Olsson-Liljequist B, Miorner H, et al (2010) Evaluating the usefulness of spa typing, in comparison with pulsed-field gel electrophoresis, for epidemiological typing of methicillin-resistant *Staphylococcus aureus* in a low-prevalence region in Sweden 2000-2004. Clin Microbiol Infect 16 (5):456-462

[27] Holtfreter S, Bauer K, Thomas D et al (2004) egc-Encoded superantigens from *Staphylococcus aureus* are neutralized by human sera much less efficiently than are classical staphylococcal enterotoxins or toxic shock syndrome toxin. Infect Immun 72 (7):4061-4071

[28] Johnson WM, Tyler SD, Ewan EP et al (1991) Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. J Clin Microbiol 29 (3):426-430

[29] Jarraud S, Mougel C, Thioulouse J et al (2002) Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. Infect Immun 70 (2):631-641

[30] Lina G, Boutite F, Tristan A et al (2003) Bacterial competition for human nasal cavity colonization: role of Staphylococcal agr alleles. Appl Environmental Microbiol 69 (1):18-23

Figure legend

Fig. 1 Characterization of the three *edin* alleles. A) PCR amplification of *edin-A*, *edin-B* and *edin-C* from *S. aureus* strains S25*edin-A(*+)[15], S7256*edin-B(*+) and S7475*edin-C(*+) (this study), respectively, using specific oligonucleotides *edinA*, *edinB*, *edinC* (Table 1) and the previously described *edin* oligonucleotides referred as *edinX* [19]. B) Sequence alignments of *edin-A*, *edin-B* and *edin-C* showing sequence homologies and localization of each oligonucleotide (underlined: *edinX*; highlighted: *edinA*, *edinB* and *edinC*).

Table 1: Oligonucleotides primers used in this study

Gene	Primer sequences	Size (kb)	References
*edin*A	Sense 5'-GGAGATATTAATAAGCTAGATTC-3'	455	This study
	Antisense 5'-ATTTTCTTTTTATCATTTGACAATTCT-3'		
*edin*B	Sense 5'-GGTGACGTGAACAAATTATCCGA-3'	455	This study
	Antisense 5'-ATCTTTCTTTTGTTATCAGAAAGTTTA-3'		
*edin*C	Sense 5'-CGCCATTAAGGTCTAGTCAAGG-3'	320	This study
	Antisense 5'-TAGGTCTTCCAGCTAATGCAGC-3'		
bbp	Sense 5'-AACTACATCTAGTACTCAACAACAG-3'	575	[25]
	Antisense 5'-ATGTGCTTGAATAACACCATCATCT-3'		
cna	Sense 5'-GTCAAGCAGTTATTAACACCAGAC-3'	423	[25]
	Antisense 5'-AATCAGTAATTGCACTTTGTCCACTG-3'		
ebpS	Sense 5'-CATCCAGAACCAATCGAAGAC-3'	186	[25]
	Antisense 5'-CTTAACAGTTACATCATCATGTTTATCTTTG-3'		
fnbA	Sense 5'-GTGAAGTTTTAGAAGGTGGAAAGATTAG -3'	643	[25]
	Antisense 5'-GCTCTTGTAAGACCATTTTTCTTCAC-3'		
fnbB	Sense 5'-GTAACAGCTAATGGTCGAATTGATACT-3'	524	[25]
	Antisense 5'-CAAGTTCGATAGGAGTACTATGTTC-3'		
fib	Sense 5'-CTACAACTACAATTGCCGTCAACAG-3'	404	[25]
	Antisense 5'-GCTCTTGTAAGACCATTTTCTTCAC-3'		
clfA	Sense 5'-ATTGGCGTGGCTTCAGTGCT-3'	292	[25]
	Antisense 5'-CGTTTCTTCCGTAGTTGCATTTG-3'		
clfB	Sense 5'-ACATCAGTAATAGTAGGGGGCAAC-3'	205	[25]
	Antisense 5'-TTCGCACTGTTTGTGTTTGCAC-3'		
eno	Sense 5'- ACGTGCAGCAGCTGACT-3'	302	[25]
	Antisense 5'- CAACAGCATYCTTCAGTACCTTC-3'		
sea	Sense 5'-GCAGGGAACAGCTTTAGGC-3'	520	[27]
	Antisense 5'-GTTCTGTAGAAGTATGAAACACG-3'		
seb	Sense 5'-ATGTAATTTTGATATTCGCAGTG-3'	683	[27]
	Antisense 5'-TGCAGGCATCATATCATACCA-3'		
sec	Sense 5'-CTTGTATGTATGGAGGAATAACAA-3'	283	[27]

	Antisense 5’-TGCAGGCATCATATCATACCA-3’		
sed	Sense 5’-GTGGTGAAATAGATAGGACTGC-3’	384	[27]
	Antisense 5’-ATATGAAGGTGCTCTGTGG-3’		
see	Sense 5’-TACCAATTAACTTGTGGATAGAC-3’	170	[27]
	Antisense 5’-CTCTTTGCACCTTACCGC-3’		
seg	Sense 5’-CGTCTCCACCTGTTGAAGG-3’	327	[27]
	Antisense 5’-CCAAGTGATTGTCTATTGTCG-3’		
seh	Sense 5’-CAACTGCTGATTTAGCTCAG-3’	360	[27]
	Antisense 5’-GTCGAATGAGTAATCTCTAGG-3’		
sei	Sense 5’-CAACTCGAATTTTCAACAGGTAC-3’	465	[27]
	Antisense 5’- CAGGCAGTCCATCTCCTG-3’		
sej	Sense 5’- CATCAGAACTGTTGTTCCGCTAG-3’	142	[27]
	Antisense 5’- CTGAATTTTACCATCAAAGGTAC-3’		
sek	Sense 5’- ATGGCGGAGTCACAGCTACT-3’	197	[27]
	Antisense 5’-TGCCGTTATGTCCATAAATGTT-3’		
seq	Sense 5’-GAACCTGAAAAGCTTCAAGGA-3’	209	[27]
	Antisense 5’-ATTCGCCAACGTAATTCCAC-3’		
eta	Sense 5’-CTAGTGCATTTGTTATTCAA-3’	119	[28]
	Antisense 5’-TGCATTGACACCATAGTACT-3’		
etb	Sense 5’-ACGGCTATATACATTCAATT-3’	200	[28]
	Antisense 5’-TCCATCGATAATATACCTAA-3’		
etd	Sense 5'-ATGACTAAAAATATATTAAAAAGTT-3’	846	This study
	Antisense 5‘-CTAATGAGACTGTAATTCAGC-3’		
lukPV	Sense 5’-ATCATTAGGTAAAATGTCTGGACATGATCCA-3’	433	[29]
	Antisense 5’-GCATCAASTGTATTGGATAGCAAAAGC-3’		
lukE	Sense 5’-TGAAAAAGGTTCAAAGTTGATACGAG-3’	269	[29]
	Antisense 5’-TGTATTCGATAGCAAAAGCAGTGCA-3’		
tst-1	Sense 5’-GCTTGCGACAACTGCTACAG-3’	559	[27]
	Antisense 5’-TGGATCCGTCATTCATTGTTAA-3’		
agr1	Sense 5’-ATGCACATGG TGCACATGC-3’	439	[30]
	Antisense 5’-GTCACAAGTACTATAAGCTGCGAT-3’		
agr2	Sense 5’-ATGCACATGG TGCACATGC-3’	572	[30]
	Antisense 5’-TATTACTAATTGAAAAGTGC CATAGC-3’		

agr3	Sense 5’-ATGCACATGG TGCACATGC-3’	321	[30]
	Antisense 5’-GTAATGTAATAGCTTGTATAATAATACCCAG-3’		
agr4	Sense 5’-ATGCACATGG TGCACATGC-3’	657	[30]
	Antisense 5’-CGATAATGCCGTAATACCCG-3’		
spa	Sense5’-TGTAAAACGACGGCCAGTTAAAGACGATCCTTCGGTGAGC-3’		[23]
	Antisense5’-CAGGAAACAGCTATGACCCAGCAGTAGTGCCGTTTGCTT-3’		

Table 2: Virulence profile and antibiotic susceptibility of clinical *edin*-positive *S. aureus* strains.

	blood (n=2)		**superficial soft tissue (n=11)**		**urine sample (n=4)**		**sputum sample (n=10)**		**deep-seated soft tissue (n=9)**		**total (n=36)**	
	n	%	n	%	n	%	n	%	n	%	n	%
Virulence profile												
MSCRAMMs												
bbp	0	0	3	27	3	75	3	30	6	67	15	42
cna	1	50	9	82	4	100	10	100	8	89	32	89
ebpS	0	0	5	45	1	25	6	60	6	67	18	50
fnbA	1	50	7	64	1	25	5	50	5	56	19	53
fnbB	1	50	11	100	4	100	7	70	8	89	31	86
fib	2	100	10	91	4	100	7	70	9	100	32	89
clfA	2	100	11	100	4	100	10	100	9	100	36	100
clfB	2	100	11	100	4	100	10	100	9	100	36	100
eno	2	100	11	100	4	100	10	100	9	100	36	100
SEs												
sea	1	50	10	91	3	75	9	90	9	100	32	89
seb	1	50	7	64	2	50	8	80	6	67	24	67
sec	1	50	7	64	2	50	10	100	6	67	26	72
sed	0	0	8	73	3	75	6	60	6	67	23	64
see	0	0	0	0	0	0	0	0	0	0	0	0
sek	1	50	5	45	1	25	7	70	2	22	16	44
seq	1	50	3	27	1	25	5	50	5	56	15	42
seg	2	100	10	91	2	50	10	100	9	100	33	92
seh	0	0	0	0	0	0	0	0	1	11	1	3
sei	2	100	11	100	2	50	9	90	8	89	32	89
sej	0	0	1	9	0	0	2	20	1	11	4	11
tst-1	2	100	6	55	1	25	7	70	5	56	21	58
etA	0	0	2	18	0	0	2	20	2	22	6	17
etB	1[&]	50	4[$]	36	0	0	2	20	1	11	8	22
etD	0	0	0	0	1*	25	1*	10	0	0	2	6
lukPV	0	0	1	9	1	25	0	0	3	33	5	14
lukE	2	100	8	73	3	75	5	50	4	44	24	67
agr group												
agr1	1	50	3	27	3	75	6	60	5	56	18	50
agr2	1	50	3	27	1	25	1	10	0	0	6	17
agr3	0	0	3	27	0	0	0	0	3	33	6	17
agr4	0	0	2	18	0	0	3	30	1	11	6	17
antibiotic resistance profile												
penicillin G	1	50	5	45	2	50	8	80	6	67	22	61
Methicillin	0	0	0	0	0	0	0	0	1	11	1	3
Erythromycin	0	0	1	9	0	0	1	10	1	11	3	8
Clindamycin	0	0	0	0	0	0	0	0	1	11	1	3
Fusidic acid	0	0	0	0	1¤	25	0	0	1¤	11	2	6

* *Edin B* positive strains
[$] *Edin A* positive for one strain
[&] *EdinA* positive strain
¤ Increase in fusidic acid MIC, classified as intermediary sensibility

Table 3 : *spa*-type of 36 *edin*-positive *Staphylococcus aureus* isolates.

Strains	EDIN type	*Spa*-type or repeat sequences	MLST
Blood			
S25	A	t6403	-
S7232	C	NT[$]	-
Deep-seated soft tissue			
S7272	C	t6953	-
S7404	C	t6649	-
S7408	C	t6484	-
S7466	C	t012	ST-30
S7595	C	NT	-
S7600	C	t6677	-
S7926	C	t6956	-
S8028*	C	NT	-
S8087	C	t6481	-
Sputum sample			
S7225	C	t2726	-
S7259	C	t2088	-
S7262	C	t6956	-
S7413	C	t6483	-
S7535	C	t2647	-
S7634	C	t6954	-
S7649	B	NT	-
S7920	C	NT	-
S7965	C	NT	-
S8100	C	t015	ST-45
Superficial soft tissue			
S7181	C	t6650	-
S7183	C	NT	-
S7436	C	t6957	-
S7475	C	t6480	-
S7526	C	t137	-

S7539	C	NT	-
S7569	A	t031	ST-45
S7599	C	t6482	-
S7932	C	NT	-
S7938	C	t620	-
S7977	C	NT	-
Urine sample			
S7256	B	t078	ST-26
S7322	C	t012	ST-30
S7906	C	t645	-
S7946	C	t216	ST-59

* MRSA strain

[$] Not typable

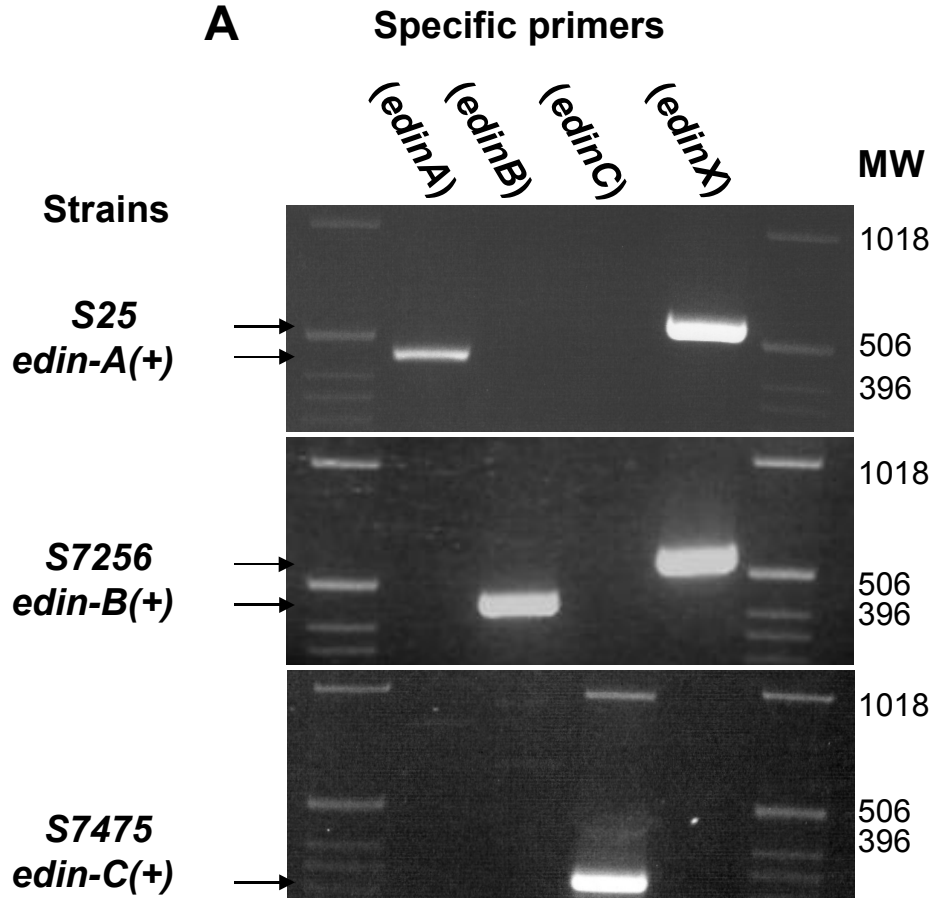
Table 4: Presence of *edin* genes in 256 *Staphylococcus aureus* strains associated with various clinical syndromes.

Source of isolates (N)	Number of *edin* isolates (%)	*edin*-isoforms		
		*edin*A	*edinB*	*edinC*
Blood (28)	2 (7.1)	1	0	1
Urine (41)	4 (9.8)	0	1	3
Superficial soft tissue (83)	11 (13.3)	1	0	10
Deep-seated soft tissue (35)	9 (25.7)*	0	0	9
Sputum (69)	10 (14.5)	0	1	9
Total (256)	**36 (14)**	**2 (5)**	**2 (5)**	**32 (90)**

* $p<0,05$ (Fisher's exact test)

Figure1

A

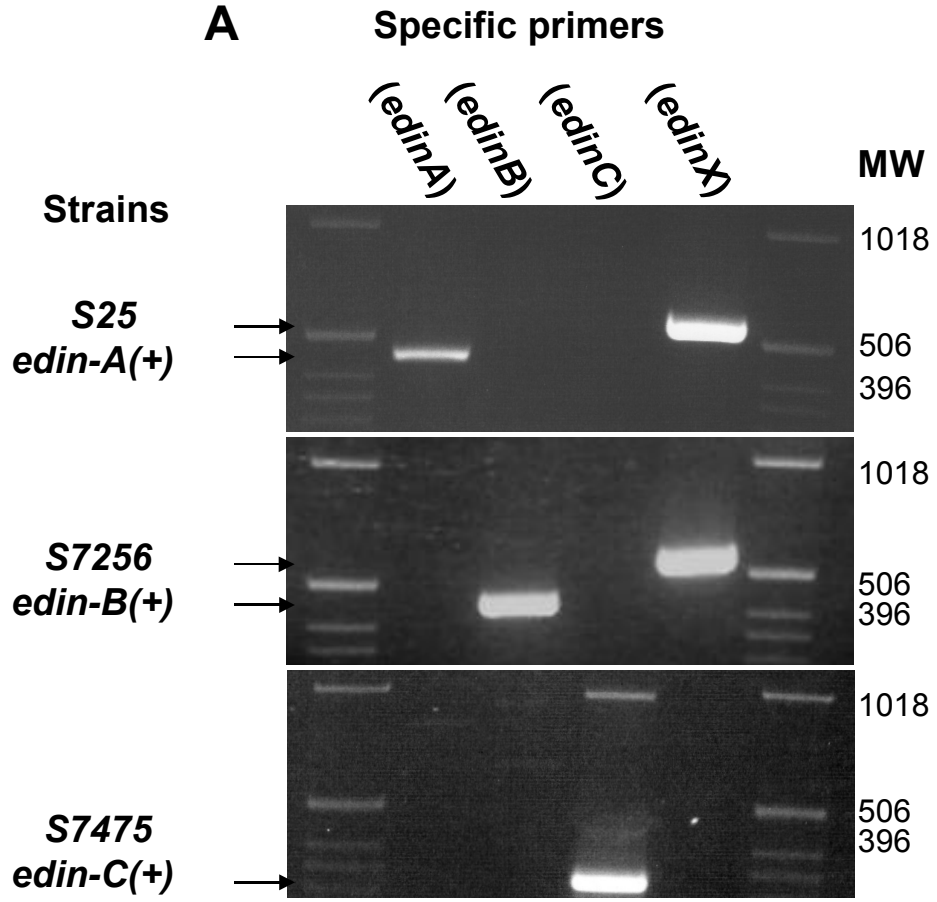


B

```
EDIN-A  ATGAAAAACAAATTACTTTTTAAAATTTTTTTGAGTTTATCTTTAGCATT
EDIN-C  ATGAAAAGAAAATTATTTTTTAAAATTATTTTTGTTTTATCTTTAGTATT
EDIN-B  ATGAAAGATA---CAATTGTAAAATTTCTATCAGCATTCCTTGTGATTTC
        ******    *    * ** * *** ** * *      **   * *    *

EDIN-A  AAGCGTTTATTCAATTAATGA----TAAAATCATAGAAGTATCTAATACT
EDIN-C  AAGCATTCATTCAATTAATGA----CAGAACTACAGAGTTATCAAACATT
EDIN-B  AAGTATTAGTTTGATAGATACATCTTTTAGCTCTAAATATAATAAA-ATC
        ***  **  **  **  **          *     * *  **    ** *

EDIN-A  TCTTTAGCAGCTGATGTTAAAAATTTCACTGATTTAGATGAGGCAACTAA
EDIN-C  GCTTTAGCAGATGATGTTAAAAATTTTACCGATTTAACTGAAGCAACTAA
EDIN-B  TCAATAGCTGCCGAGACTAAAAATTTTACAGACTTAGTTGAAGCTACTAA
         *  **** *  **   ********* ** ** ***  *** ** *****

EDIN-A  ATGGGGGAATAAACTTATAAAACAAGCTAAGTATAGTTCGGATGATAAAA
EDIN-C  CTGGGGTAATAAGCTTATAAAACAAGCTAATTACAGTTCAAAAGACAAAG
EDIN-B  ATGGGGAAACTCATTAATAAAGTCAGCCAAGTATTCTTCTAAAGATAAGA
         ***** **     * *****   *** ** **   ***  * ** **

EDIN-A  TAGCTCTATACGAATATACAAAAGATAGTTCTAAGATAAATGGTCCATTA
EDIN-C  AAGCTATTTATAATTATACAAAATATAGCTCGCCTATAAATACGCCATTA
EDIN-B  TGGCTATTTATAATTATACAAAAAATAGTTCACCCATAAATACTCCTCTA
          *** * **  * ********* **** **    ******   **  **

EDIN-A  AGACTCGCAGGTGGAGATATTAATAAGCTAGATTCAACAACTCAAGACAA
EDIN-C  AGGTCTAGTCAAGGTGATATAAGTAATTTTTCTGCAGATTTACAAGAAAA
EDIN-B  AGATCAGCAAATGGTGACGTGAACAAATTATCCGAAAACATTCAAGAGCA
        **          ** **  * *  **  *      *      *****  *

EDIN-A  AGTAAGAAGATTAGATTCATCTATTTCTAAATCTACTACTCCTGAATCTG
EDIN-C  AATACTTCGATTAGATAGACTCATAAGCAAATCAAGTACTAGTGATTCTG
EDIN-B  GGTTAGACAATTAGACTCAACGATATCTAAATCTGTAACACCAGATTCAG
          *      ******   *   **    *****    **    ** ** *

EDIN-A  TATACGTTTATAGACTTTTAAATTTAGATTATTTGACAAGTATCGTTGGA
EDIN-C  TATATGTTTATAGATTGCTAAATCTGGACTATTTATCCAGTGTTAAAGGT
EDIN-B  TCTATGTATATAGATTATTAAATTTAGACTACTTATCAAGTATAACTGGC
        * ** ** ****** *  ***** * ** ** **  * *** *    **

EDIN-A  TTTACAAATGAAGATTTATATAAATTACAACAGACCAATAATGGCCAGTA
EDIN-C  TTTTCTTCTGAAGATTTGGAATTATTATACAAAACAGAAAATGGTAAGTA
EDIN-B  TTTACGCGAGAAGATTTACATATGCTACAACAAACTAACAATGGTCAATA
        *** *    ********  *     ** *  * **  * *****  * **

EDIN-A  TGATGAAAATCTAGTTAGAAAGCTTAATAACGTTATGAATAGCAGAATAT
EDIN-C  TAATGAAGAATTAGTTAAAAAACTTAATAATATTATGAATAGTAAAATTT
EDIN-B  TGATGAAGCGCTTGTGTCAAAACTAAATAATCTTATGAATAGTAGAATTT
        * *****    * **   *** ** *****  ********** * *** *

EDIN-A  ATAGAGAAGACGGATACTCTAGTACACAATTAGTTAGTGGAGCAGCTGTA
EDIN-C  ATACTGAGTACGGTTATTCTAGCACTCAATTAGTTAAAGGAGCTGCATTA
EDIN-B  ACAGAGAAAATGGCTACTCTAGTACACAACTAGTTAGTGGTGCAGCACTA
        * *  **  * ** ** ***** ** *** ******  ** ** **  **

EDIN-A  GGTGGTAGACCTATTGAATTAAGGTTAGAATTACCAAAAGGGACTAAAGC
EDIN-C  GCTGGAAGACCTATTGAATTGAAATTACAATTACCAAAAGGTACTAAAGC
EDIN-B  GCAGGTAGGCCAATTGAATTAAAATTAGAATTACCTAAAGGTACTAAAGC
        *  ** ** ** ******** *  *** ******* ***** ********

EDIN-A  TGCGTATCTTAATTCTAAAGATTTAACTGCTTACTATGGTCAACAAGAAG
EDIN-C  TGCCTATATCGATTCTAAAAATCTTACTGCATATCCCGGACAACAAGAAA
EDIN-B  AGCATATATTGATTCTAAAGAGTTAACAGCATACCCAGGTCAACAAGAAG
         ** *** *  ******** *  * ** ** **     ** *********

EDIN-A  TTTTATTACCTAGAGGCACAGAATACGCTGTTGGAAGTGTAGAATTGTCA
EDIN-C  TATTGTTGCCTAGAGGAACAGACTACACTATAAATACAGTCAAACTTTCA
EDIN-B  TTCTTTTGCCTAGAGGGACAGAATATGCTGTAGGCAGTGTTAAACTTTCT
        *  * ** ******** ***** **  ** *    *  **  ** * **

EDIN-A  AATGATAAAAAGAAAATCATAATAACAGCTATTGTTTTTAAAAAATAG
EDIN-C  GATGATCATAAAAGAATTTTAATCGAAGGTATCGTTTTCAAAAAGTAA
EDIN-B  GATAACAAAAGAAAGATAATTATAACTGCTGTAGTTTTTAAAAAATAA
         ** *  * *  *  **  * **    * * * ***** ***** **
```

Figure1

A



B

```
EDIN-A  ATGAAAAACAAATTACTTTTTAAAATTTTTTTGAGTTTATCTTTAGCATT
EDIN-C  ATGAAAAGAAAATTATTTTTTAAAATTATTTTTGTTTTATCTTTAGTATT
EDIN-B  ATGAAAGATA---CAATTGTAAAATTTCTATCAGCATTCCTTGTGATTTC
        ******    *    * ** * *** ** * *      **   * *    *

EDIN-A  AAGCGTTTATTCAATTAATGA----TAAAATCATAGAAGTATCTAATACT
EDIN-C  AAGCATTCATTCAATTAATGA----CAGAACTACAGAGTTATCAAACATT
EDIN-B  AAGTATTAGTTTGATAGATACATCTTTTAGCTCTAAATATAATAAA-ATC
        ***  **  **  **  **          *     * *  **    ** *

EDIN-A  TCTTTAGCAGCTGATGTTAAAAATTTCACTGATTTAGATGAGGCAACTAA
EDIN-C  GCTTTAGCAGATGATGTTAAAAATTTTACCGATTTAACTGAAGCAACTAA
EDIN-B  TCAATAGCTGCCGAGACTAAAAATTTTACAGACTTAGTTGAAGCTACTAA
         *  **** *  **   ********* ** ** ***  *** ** *****

EDIN-A  ATGGGGGAATAAACTTATAAAACAAGCTAAGTATAGTTCGGATGATAAAA
EDIN-C  CTGGGGTAATAAGCTTATAAAACAAGCTAATTACAGTTCAAAAGACAAAG
EDIN-B  ATGGGGAAACTCATTAATAAAGTCAGCCAAGTATTCTTCTAAAGATAAGA
         ***** **     * *****   *** ** **   ***  * ** **

EDIN-A  TAGCTCTATACGAATATACAAAAGATAGTTCTAAGATAAATGGTCCATTA
EDIN-C  AAGCTATTTATAATTATACAAAATATAGCTCGCCTATAAATACGCCATTA
EDIN-B  TGGCTATTTATAATTATACAAAAAATAGTTCACCCATAAATACTCCTCTA
          *** * **  * ********* **** **    ******   **  **

EDIN-A  AGACTCGCAGGTGGAGATATTAATAAGCTAGATTCAACAACTCAAGACAA
EDIN-C  AGGTCTAGTCAAGGTGATATAAGTAATTTTTCTGCAGATTTACAAGAAAA
EDIN-B  AGATCAGCAAATGGTGACGTGAACAAATTATCCGAAAACATTCAAGAGCA
        **          ** **  * *  **  *      *      *****  *

EDIN-A  AGTAAGAAGATTAGATTCATCTATTTCTAAATCTACTACTCCTGAATCTG
EDIN-C  AATACTTCGATTAGATAGACTCATAAGCAAATCAAGTACTAGTGATTCTG
EDIN-B  GGTTAGACAATTAGACTCAACGATATCTAAATCTGTAACACCAGATTCAG
          *      ******   *   **    *****    **    ** ** *

EDIN-A  TATACGTTTATAGACTTTTAAATTTAGATTATTTGACAAGTATCGTTGGA
EDIN-C  TATATGTTTATAGATTGCTAAATCTGGACTATTTATCCAGTGTTAAAGGT
EDIN-B  TCTATGTATATAGATTATTAAATTTAGACTACTTATCAAGTATAACTGGC
        * ** ** ****** *  ***** * ** ** **  * *** *    **

EDIN-A  TTTACAAATGAAGATTTATATAAATTACAACAGACCAATAATGGCCAGTA
EDIN-C  TTTTCTTCTGAAGATTTGGAATTATTATACAAAACAGAAAATGGTAAGTA
EDIN-B  TTTACGCGAGAAGATTTACATATGCTACAACAAACTAACAATGGTCAATA
        *** *    ********  *     ** *  * **  * *****  * **

EDIN-A  TGATGAAAATCTAGTTAGAAAGCTTAATAACGTTATGAATAGCAGAATAT
EDIN-C  TAATGAAGAATTAGTTAAAAAACTTAATAATATTATGAATAGTAAAATTT
EDIN-B  TGATGAAGCGCTTGTGTCAAAACTAAATAATCTTATGAATAGTAGAATTT
        * *****    * **   *** ** *****  ********** * *** *

EDIN-A  ATAGAGAAGACGGATACTCTAGTACACAATTAGTTAGTGGAGCAGCTGTA
EDIN-C  ATACTGAGTACGGTTATTCTAGCACTCAATTAGTTAAAGGAGCTGCATTA
EDIN-B  ACAGAGAAAATGGCTACTCTAGTACACAACTAGTTAGTGGTGCAGCACTA
        * *  **  * ** ** ***** ** *** ******  ** ** **  **

EDIN-A  GGTGGTAGACCTATTGAATTAAGGTTAGAATTACCAAAAGGGACTAAAGC
EDIN-C  GCTGGAAGACCTATTGAATTGAAATTACAATTACCAAAAGGTACTAAAGC
EDIN-B  GCAGGTAGGCCAATTGAATTAAAATTAGAATTACCTAAAGGTACTAAAGC
        *  ** ** ** ******** *  *** ******* ***** ********

EDIN-A  TGCGTATCTTAATTCTAAAGATTTAACTGCTTACTATGGTCAACAAGAAG
EDIN-C  TGCCTATATCGATTCTAAAAATCTTACTGCATATCCCGGACAACAAGAAA
EDIN-B  AGCATATATTGATTCTAAAGAGTTAACAGCATACCCAGGTCAACAAGAAG
         ** *** *  ******** *  * ** ** **     ** *********

EDIN-A  TTTTATTACCTAGAGGCACAGAATACGCTGTTGGAAGTGTAGAATTGTCA
EDIN-C  TATTGTTGCCTAGAGGAACAGACTACACTATAAATACAGTCAAACTTTCA
EDIN-B  TTCTTTTGCCTAGAGGGACAGAATATGCTGTAGGCAGTGTTAAACTTTCT
        *  * ** ******** ***** **  ** *    *  **  ** * **

EDIN-A  AATGATAAAAAGAAAATCATAATAACAGCTATTGTTTTTAAAAAATAG
EDIN-C  GATGATCATAAAAGAATTTTAATCGAAGGTATCGTTTTCAAAAAGTAA
EDIN-B  GATAACAAAAGAAAGATAATTATAACTGCTGTAGTTTTTAAAAAATAA
         ** *  * *  *  **  * **    * * * ***** ***** **
```